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(54) Title: PROTEASE VARIANTS AND COMPOSITIONS

(57) Abstract

A subtilisin having improved storage stability or improved performance in detergents, wherein one or more amino acid residues situated in, or in the vicinity of a hydrophobic domain of the parent subtilase have been substituted for an amino acid residue more hydrophobic than the original residue, said hydrophobic domain comprising the residues corresponding to residues P129, P131, I165, Y167, Y171 or BLS309 (in BASBPN numbering), and said residues in the vicinity thereof comprises residues corresponding to the residues E136, G159, S164, R170, A194, and G195 of BLS309 (in BASBPN numbering), with the exception of the R170M, R170I and R170Y variant of BLS309.

is equivalent
is 5837517

substitution
listing
in p30, p65

p32 missing!
discloses
Y171Q

Title: Protease Variants and Compositions

TECHNICAL FIELD

This invention relates to novel mutant enzymes or enzyme variants useful in formulating detergent compositions and exhibiting improved storage stability while retaining or improving their wash performance; cleaning and detergent compositions containing said enzymes; mutated genes coding for the expression of said enzymes when inserted into a suitable host cell or organism; and such host cells transformed therewith and capable of expressing said enzyme variants.

BACKGROUND OF THE INVENTION

In the detergent industry enzymes have for more than 30 years been implemented in washing formulations. Enzymes used in such formulations comprise proteases, lipases, amylases, cellulases, as well as other enzymes, or mixtures thereof. Commercially most important are proteases.

Although proteases have been used in the detergent industry for more than 30 years, much remains unknown as to details of how these enzymes interact with substrates and/other substances present in *e.g.* detergent compositions. Some factors related to specific residues and influencing certain properties, such as oxidative and thermal stability in general have been elucidated, but much remains to be found out. Also, it is still not exactly known which physical or chemical characteristics are responsible for a good washing performance or stability of a protease in a specific detergent composition.

"*Principles of Biochemistry*," Fifth Edition, McGraw-Hill Book Company, NY, pp. 271-272).

The bacterial serine proteases have molecular weights in the 20,000 to 45,000 Daltons range. They are inhibited by diisopropylfluorophosphate. They hydrolyze simple terminal esters and are similar in activity to eukaryotic chymotrypsin, also a serine protease. A more narrow term, alkaline protease, covering a sub-group, reflects the high pH optimum of some of the serine proteases, from pH 9.0 to 11.0 (for review, see Priest (1977) *Bacteriological Rev.* **41** 711-753).

A sub-group of the serine proteases tentatively designated subtilases has been proposed by Siezen *et al.*, *Protein Engng.* **4** (1991) 719-737. They are defined by homology analysis of more than 40 amino acid sequences of serine proteases previously referred to as subtilisin-like proteases. A subtilisin was previously defined as a serine protease produced by Gram-positive bacteria or fungi, and according to Siezen *et al.* now is a subgroup of the subtilases. A wide variety of subtilisins have been identified, and the amino acid sequence of a number of subtilisins have been determined. These include more than six subtilisins from *Bacillus* strains, namely, subtilisin 168, subtilisin BPN', subtilisin Carlsberg, subtilisin Y, subtilisin amylosacchariticus, and mesentericopeptidase (Kurihara *et al.* (1972) *J. Biol. Chem.* **247** 5629-5631; Wells *et al.* (1983) *Nucleic Acids Res.* **11** 7911-7925; Stahl and Ferrari (1984) *J. Bacteriol.* **159** 811-819, Jacobs *et al.* (1985) *Nucl. Acids Res.* **13** 8913-8926; Nedkov *et al.* (1985) *Biol. Chem. Hoppe-Seyler* **366** 421-430, Svendsen *et al.* (1986) *FEBS Lett.* **196** 228-232), one subtilisin from an actinomycetales, thermitase from *Thermoactinomyces vulgaris* (Meloun *et al.* (1985) *FEBS Lett.* **198** 195-200), and one fungal subtilisin, proteinase K from *Tritirachium album* (Jany and Mayer (1985) *Biol.Chem.*

In the context of this invention, a subtilase variant or mutated subtilase means a subtilase that has been produced by an organism which is expressing a mutant gene derived from a parent microorganism which possessed an original or parent gene and which produced a corresponding parent enzyme, the parent gene having been mutated in order to produce the mutant gene from which said mutated subtilisin protease is produced when expressed in a suitable host.

Random and site-directed mutations of the subtilase gene have both arisen from knowledge of the physical and chemical properties of the enzyme and contributed information relating to subtilase's catalytic activity, substrate specificity, tertiary structure, etc. (Wells *et al.* (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**; 1219-1223; Wells *et al.* (1986) *Phil. Trans. R. Soc.* Lond.A. **317** 415-423; Hwang and Warshel (1987) *Biochem.* **26** 2669-2673; Rao *et al.*, (1987) *Nature* **328** 551-554.

More recent publications covering this area are Carter *et al.* (1989) *Proteins* **6** 240-248 relating to design of variants that cleave a specific target sequence in a substrate (positions 24 and 64); Graycar *et al.* (1992) *Annals of the New York Academy of Sciences* **672** 71-79 discussing a number of previously published results; and Takagi (1993) *Int. J. Biochem.* **25** 307-312 also reviewing previous results.

Especially site-directed mutagenesis of the subtilisin genes has attracted much attention, and various mutations are described in the following patent applications and patents:

EP publ. no. 130756 (GENENTECH)(corresponding to US Reissue Patent No. 34,606 (GENENCOR)) relating to site specific or randomly generated mutations in "carbonyl hydrolases" and subsequent screening of the mutated enzymes for various properties, such as k_{cat}/K_m ratio, pH-activity profile, and

with the inventors knowledge of the tertiary structure of subtilisin BPN' lead the inventors to select a number of positions susceptible to mutation with an expectation of obtaining mutants with altered properties. The positions so identified are: ^{124}Met, ^{222}Met, ^{104}Tyr, ^{152}Ala, ^{156}Glu, ^{166}Gly, ^{169}Gly, ^{189}Phe, ^{217}Tyr. Also ^{155}Asn, ^{21}Tyr, ^{22}Thr, ^{24}Ser, ^{32}Asp, ^{33}Ser, ^{36}Asp, ^{46}Gly, ^{48}Ala, ^{49}Ser, ^{50}Met, ^{77}Asn, ^{87}Ser, ^{94}Lys, ^{95}Val, ^{96}Leu, ^{107}Ile, ^{110}Gly, ^{170}Lys, ^{171}Tyr, ^{172}Pro, ^{197}Asp, ^{199}Met, ^{204}Ser, ^{213}Lys, and ^{221}Ser, which positions are identified as being expected to influence various properties of the enzyme. Also, a number of mutations are exemplified to support these suggestions. In addition to single mutations in these positions the inventors also performed a number of multiple mutations. Further the inventors identify ^{215}Gly, ^{67}His, ^{126}Leu, ^{135}Leu, and amino acid residues within the segments 97-103, 126-129, 213-215, and 152-172 as having interest, but mutations in any of these positions are not exemplified.

Especially of interest for the purpose of the present invention the inventors of EP 251 446 suggest to substitute ^{170}Lys (in subtilisin BPN', type I-S1), specifically they suggest to introduce Glu or Arg for the original Lys. It appears that the Glu variant was produced and it was found that it was highly susceptible to autolytic degradation (cf. pages 48, 121, 123 (Table XXI includes an obvious error, but indicates a reduction in autolysis half-time from 86 to 13 minutes) and Fig. 32).

EP publ. no. 260105 (GENENCOR) describes modification of certain properties in enzymes containing a catalytic triad by selecting an amino acid residue within about 15 Å from the catalytic triad and replace the selected amino acid residue with another residue. Enzymes of the subtilase type described in the present specification are specifically mentioned as belonging to the class of enzymes containing a catalytic triad.

S024, S019, S020, S203, S225, S227 in the same Table and Table VII) all in accordance with the generic concept of said application.

In EP 525 610 A1 (SOLVAY) it is suggested to improve the stability of the enzyme (a type I-S2 subtilase closely related to subtilisin PB92) towards ionic tensides by decreasing the hydrophobicity in certain surface regions thereof. It is consequently suggested to substitute Gln for the Arg in position 164 (170 if using BPN' numbering). No variants comprising this substitution are disclosed in the application.

In WO 94/02618 (GIST-BROCADES N.V.) a number of position 164 (170 if using BPN' numbering) variants of the I-S2 type subtilisin PB92 are described. Examples are provided showing substitution of Met, Val, Tyr, Ile, for the original Arg. Wash performance testing in powder detergents of the variants indicates a slight improvement. Especially for the Ile variant wash performance tests on cacao an improvement of about 20-30% is indicated. No stability data are provided.

In WO 95/30011, WO 95/30010, and WO 95/29979 (PROCTER & GAMBLE COMPANY) describe 6 regions, especially position 199-220 (BPN` numbering), in both Subtilisin BPN` and subtilisin 309, which are designed to change (i.e. decrease) the adsorption of the enzyme to surface-bound soils. It is suggested that decreased adsorption by an enzyme to a substrate results in better detergent cleaning performance. No specific detergent wash performance data are provided for the suggested variants.

WO 95/27049 (SOLVAY S.A.) describes a subtilisin 309 type protease with following mutations: N43R+N116R+N117R (BPN` numbering. Data indicate the corresponding variant is having improved stability, compared to wildtype.

To be effective, however, such enzymes must not only exhibit activity under washing conditions, but must also be compatible with other detergent components during detergent production and storage.

For example, subtilisins may be used in combination with other enzymes active against other substrates, and the selected subtilisin should possess stability towards such enzymes, and also the selected subtilisin preferably should not catalyse degradation of the other enzymes. Also, the chosen subtilisin should be resistant to the action from other components in the detergent formulation, such as bleaching agents, oxidizing agents, etc., in particular an enzyme to be used in a detergent formulation should be stable with respect to the oxidizing power, calcium binding properties, and pH conditions rendered by the non-enzymatic components in the detergent during storage and in the wash liquor during wash.

The ability of an enzyme to catalyze the degradation of various naturally occurring substrates present on the objects to be cleaned during *e.g.* wash is often referred to as its washing ability, washability, detergency, or wash performance. Throughout this application the term wash performance will be used to encompass this property.

The ability of an enzyme to remain active in the presence of other components of a detergent composition prior to being put to use (normally by adding water in the washing process) is usually referred to as storage stability or shelf life. It is often measured as half-life, $t_{½}$. We will use the expression storage stability for this property throughout this application to encompass this property.

Naturally occurring subtilisins have been found to possess properties which are highly variable in relation to their

correspond to a large extend to the classical method of isolating native enzymes, submit them to classical mutagenesis programs (using radiation or chemical mutagens) and screen them for their properties. The difference lies in that these methods are more efficient through the knowledge of the presence of a large number of variant enzymes substituted in a specific position.

A subtilisin enzyme typically comprises about 275 amino acid residues. Each residue is capable of being 1 out of 20 possible naturally occurring amino acids.

Therefore one very serious draw-back in that procedure is the very large number of mutations generated that have to be submitted to a number of preliminary screenings to determine their properties.

A procedure as outlined in these patent applications will consequently only be slightly better than the traditional random mutation procedures which have been known for years.

The other known techniques relate to changing specific properties, such as oxidation stability, thermal stability, Ca-stability, transesterification and hydrolysis rate (EP publ. no. 260105 (GENENCOR)), pH-activity profile (Thomas, Russell, and Fersht, *supra*), and substrate specificity (International patent publ. no. WO 88/07578 (GENENTECH)). None of these publications relates to changing either the wash performance of enzymes or their storage stability.

In International Patent Application no. PCT/DK 88/00002 (NOVO NORDISK A/S) it is proposed to use the concept of homology comparison to determine which amino acid positions should be selected for mutation and which amino acids should be

SUMMARY OF THE INVENTION

It has now surprisingly been found that a subtilase variant having improved storage stability and/or improved performance in detergents, can be obtained by substituting one or more amino acid residues situated in, or in the vicinity of a hydrophobic domain of the parent subtilase for an amino acid residue more hydrophobic than the original residue, said hydrophobic domain comprising the residues corresponding to residues P129, P131, I165, Y167, Y171 of BLS309 (in BASBPN numbering), and said residues in the vicinity thereof comprises residues corresponding to the residues E136, G159, S164, R170, A194, and G195 of BLS309 (in BASBPN numbering), with the exception of the R170M, R170I and R170V variants of BABP92.

The present invention relates consequently in its first aspect to enzyme variants exhibiting improved stability and/or improved wash performance in detergent.

In its second aspect the invention relates to DNA constructs capable of expressing the enzymes of the first aspect, when inserted in a suitable manner into a host cell that subsequently is brought to express the subtilisin enzyme(s) of the first aspect.

In a third aspect the invention relates to the production of the subtilisin enzymes of the invention by inserting a DNA construct according to the second aspect into a suitable host, cultivating the host to express the desired subtilase enzyme, and recovering the enzyme product.

ABBREVIATIONS

AMINO ACIDS

A	=	Ala	=	Alanine
V	=	Val	=	Valine
L	=	Leu	=	Leucine
I	=	Ile	=	Isoleucine
P	=	Pro	=	Proline
F	=	Phe	=	Phenylalanine
W	=	Trp	=	Tryptophan
M	=	Met	=	Methionine
G	=	Gly	=	Glycine
S	=	Ser	=	Serine
T	=	Thr	=	Threonine
C	=	Cys	=	Cysteine
Y	=	Tyr	=	Tyrosine
N	=	Asn	=	Asparagine
Q	=	Gln	=	Glutamine
D	=	Asp	=	Aspartic Acid
E	=	Glu	=	Glutamic Acid
K	=	Lys	=	Lysine
R	=	Arg	=	Arginine
H	=	His	=	Histidine
X	=	Xaa	=	Any amino acid

NUCLEIC ACID BASES

A	=	Adenine
G	=	Guanine
C	=	Cytosine
T	=	Thymine (only in DNA)
U	=	Uracil (only in RNA)

VARIANTS

In describing the various enzyme variants produced or contemplated according to the invention, the following nomenclatures have been adapted for ease of reference:

POSITIONS.

In describing the variants in this application and in the appended claims use is made of the alignment of various subtilases in Siezen *et al.*, *Supra*. In other publications relating to subtilases other alignments or the numbering of specific enzymes have been used. It is a routine matter for the skilled person to establish the position of a specific residue in the numbering used here. Reference is also made to Fig. 1 showing an alignment of residues relevant for the present invention from a large number of subtilases. Reference is also made to Table I of WO 91/00345 showing an alignment of residues relevant for the present invention from a number of subtilases.

TABLE I

Presently established Subtilases (from Siezen *et al.*, *supra*)

Organism	cDNA, gene	enzyme	acronym
PROKARYOTES			
Bacteria: Gram-positive			
Bacillus subtilis 168	apr A	subtilisin I168,apr	ABSS168
Bacillus amyloliquefaciens	apr	subtilisinBPN'(NOVO)	BASBPN
Bacillus subtilis DY	-	subtilisin DY	BSSDY
Bacillus licheniformis	+	subtilisin Carlsberg	BLSCAR
Bacillus lentus	+	subtilisin 147	BLS147
Bacillus alcalophilus PB92	+	subtilisin PB92	BAPB92
Bacillus sp. DSM 4828	-	alkaline protease	BDSM48
Bacillus YaB	ale	alkaline elastase YaB	BYSYAB
Bacillus subtilis 168	epr	min. extracell. prot.	BSEPR
Bacillus subtilis	bpf	bacillopeptidase F	BSBPF
Bacillus subtilis IFO3013	ispl	intracell.ser. prot.1	BSISP1
Bacillus subtilis A50	-	intracell.ser. prot.	BSIA50
Bacillus thuringiensis	-	extracell. ser. prot.	BTFINI

Organism	cDNA, gene	enzyme	acronym
HIGHER EUKARYOTES			
Worms			
Caenorhabditis elegans	bli4	cuticle protease	CEBLI4
Insects			
Drosophila (fruit fly)	fur1	furin 1	DMFUR1
Drosophila (fruit fly)	fur2	furin 2	DMFUR2
Plants			
Cucumis melo (melon)	-	cucumisin	CMCUCU
Mammals			
Human (also rat, mouse)	fur	furin	HSFURI
Human (also mouse)	+	insulinoma PC2 prot.	HSIPC2
Mouse	+	pituitary PC3 prot.	MMPPC3
Human	+	tripeptidyl peptid.II	HSTPP

References used for Table I

References to amino acid sequences (GenBank®/EMBL Data Bank accession numbers are shown in brackets):

ARB172 Kamekura and Seno, (1990) *Biochem. Cell Biol.* 68 352-359 (amino acid sequencing of mature protease residues 1-35; residue I4 not determined).

BSS168 Stahl. and Ferrari. (1984) *J. Bacteriol.* 158, 411-418 (K01988). Yoshimoto, Oyama *et al.* (I488) *J. Biochem.* **103**, 1060-1065 (the mature subtilisin from *B.subtilis* var. *amylosacchariticus* differs in having T130S and T162S). Svendsen, *et al.* (1986) *FEBS Lett.* **196**, 228-232 (PIR A23624; amino acid sequencing; the mature alkaline mesentericopeptidase From *B. mesentericus* differs in having S85A, A88S, S89A. S183A and N259S).

BSBPF	Sloma *et al.* (1990) *J. Bacteriol.* **172** 1470-1477 (M29035; corrected). Wu *et al.* (1990) *J. Biol.Chem.* **265** 6845-6850 (J05400; this sequence differs in having A169V and 586 less C-terminal residues due to a frameshift).
BSISP1	Koide *et al.* (1986) *J. Bacteriol.* **167** 110-116 (M13760).
BSIA50	Strongin *et al.* (1978) *J. Bacteriol.* **133** 1401-1411 (amino acid sequencing of mature protease residues 1-54; residues 3. 39, 40. 45, 46, 49 and 50 not determined).
BTFINI	Chestukhina *et al.* (1985) *Biokhimiya* **50** 1724-1730 (amino acid sequencing of mature protease residues 1-14 from *B. thuringiensis* variety *israeliensis*, and residues 1-16 and 223-243 from variety *finitimus*). Kunitate *et al.* (1989) *Agric. Biol. Chem.* **53** 3251-3256 (amino acid sequencing of mature protease residues 6-20 from variety *kurstaki*. BTKURS).
BCESPR	Chestukhina *et al.* (1985) *Biokhimiya* **50** 1724-1730 (amino acid sequencing of mature residues 1-16 and 223-243).
NDAPII	Tsujibo *et al.* (1990) *Agric. Biol. Chem.* **54** 2177-2179 (amino acid sequencing of mature residues 1-26).
TVTHER	Meloun *et al.* (1985) *FEBS Lett.* **183** 195-200 (PIR A00973; amino acid sequencing of mature protease residues 1-274).
EFCYLA	Segarra *et al.* (1991) *Infect. Immun.* **59** 1239-1246.
SEEPIP	Schnell *et al.* (1991) personal communication (Siezen *et al.* (*supra*)).
SPSCPA	Chen *et al.* (1990) *J. Biol. Chem.* **265** 3161-3167 (J05224).

TAPROR	Samal *et al.* (1990) *Mol. Microbiol.* **4** 1789-1792 (X56116).
TAPROT	Samal *et al.* (1989) *Gene* **85** 329-333.
AOALPR	Tatsumi *et al.* (1989) *Mol. Gen. Genet.* **219** 33-38. Cheevadhanarah *et al.* (1991) EMBL Data Library (X54726).
MPTHMY	Gaucher and Stevenson (1976) *Methods Enzymol.* **45** 415-433 (amino acid sequencing of residues 1-28,and hexapeptide LSGTSM with active site serine).
ACALPR	Isogai *et al.* (1991) *Agric. Biol. Chem.* **55** 471-477. Stepanov *et al.* (1986) *Int. J. Biochem.* **18** 369-375 (amino acid sequencing of residues 1-27: the mature protease differs in having H13[1]Q, R13[2]N and S13[6]A).
KLKEX1	Tanguy-Rougeau, Wesolowski-Louvel and Fukuhara (1988) *FEBS lett.* **234** 464-470 (X07038).
SCKEX2	Mizuno *et al.* (1988) *Biochem. Biophys. Res. Commun.* **156** 246-254(M24201).
SCPRB1	Moehle *et al.* (1987) *Mol. Cell. Biol.* **7** 4390-4399 (M18097).
YLXYPR2	Davidow *et al.* (1987) *J. Bacteriol.* **169** 4621-4629 (M17741). Matoba *et al.* (1988) *Mol. Cell Biol.* **8** 4904-4916 (M23353).
CEBL14	Peters and Rose (1991) *The Worm Breeder's Gazette* **11** 28.
DMFUR1	Roebroek *et al.* (1991) *FEBS Lett.* **289** 133-137 (X59384).
DMFUR2	Roebroek *et al.* (1992) **267** 17208-17215.
CMCUCU	Kaneda *et al.* (1984) *J. Biochem.* **95** 825-829 (amino acid sequencing of octapeptide NIISGTSM with active site serine).
HSFURI	van den Ouweland *et al.* (1990) *Nucl. Acids Res.* **18** 664 (X04329) (the sequence of mouse furin differs in 51 positions, including five in the catalytic

P129, P131, I165, Y167, Y171 of subtilisin 309 are substituted for a more hydrophobic residue. The residues in question are especially E136, G159, S164, R170, A194, and G195.

Further, said variant exhibits a particularly high improved stability in liquid detergents and in detergents in a shaped solid form.

Fig. 2 shows the hydrophobic domain in subtilisin 309 and residues in the vicinity thereof a number of which are to be substituted in order to increase the hydrophobicity of the domain. This may be achieved by substituting hydrophobic residues for non-hydrophobic residues and/or by substituting residues to become even more hydrophobic than in the parent enzyme.

The same principle applies to the corresponding domain in other subtilases, the identification of which is within the skills of the average person working in this technical field. Graphic representations like the one in Fig. 2 can be produced for other subtilases to determine the target residues to be substituted according to the invention.

A number hereof is indicated in Table II below:

enzyme, especially such hydrophobic residues that comprise a relatively long hydrophobic side chain, such as Ile, Leu, and Val, whereby, when the mutated gene is expressed, the amino acid residue is substituted by a more hydrophobic residue, which increases the hydrophobicity of the domain as such.

Hydrophobic amino acid residues are generally the following: Val (V), Ile (I), Leu (L), Met (M), Phe (F), Pro (P) and Trp(W). Among these Val, Ile and Leu are preferred.

By looking at Table II and applying the principle of the invention a number of candidates for substitution becomes clear.

For both BASBPN and BLSCAR it seems appropriate to make substitutions in positions 129, 131, 136, 159, 164, 167, 170, 171 and 195. In BLS309 positions 136, 164, 167, and 170, 171 would be the first choices, and positions 159 and 195 also would be a second choice. In BLS147 positions 129, 131, 136, 167, 170, 171 and 195 are the first choice, while positions 159 and 164 are second. Finally, in TVTHER positions 129, 131, 136, 167, 171 and 194 are the first choices, with 164 as a second one.

According to the invention it would entail an advantage to substitute the Gly residues in the hydrophobic domain to bulkier and more hydrophobic residues.

Such considerations apply for any hydrophilic or hydrophobic residue that may occupy any of the above mentioned position, meaning that any increase in hydrophobicity seems to be advantageous. This means that *e.g.* a very hydrophilic residue such as the charged residues Arg (R), Asp (D), Glu (E) or Lys (K) may be substituted by any residue that is less hydrophilic. Such less hydrophilic residues comprises the residues Gly (G),

VARIANTS

A: Single variants:

Subtilisin BPN', Subtilisin Carlsberg, Subtilisin 168, and Subtilisin DY variants:

A129V, A129I, A129L, A129M, A129F
G131V, G131I, G131L, G131M, G131F
K136V, K136I, K136L, K136M, K136F,
S159V, S159I, S159L, S159M, S159F,
T164V, T164I, T164L, T164M, T164F,
Y167V, Y167I, Y167L, Y167M, Y167F
K170V, K170I, K170L, K170M, K170F,
Y171V, Y171I, Y171L, Y171M, Y171F
A194V, A194I, A194L, A194M, A194F
E195V, E195I, E195L, E195M, E195F,

Thermitase variants:

A129V, A129I, A129L, A129M, A129F
G131V, G131I, G131L, G131M, G131F
Q136V, Q136I, Q136L, Q136M, Q136F,
T159V, T159I, T159L, T159M, T159F,
A164V, A164I, A164L, A164M, A164F,
Y167V, Y167I, Y167L, Y167M, Y167F
Y171V, Y171I, Y171L, Y171M, Y171F
Y170V, Y170I, Y170L, Y170M, Y170F,
S194V, S194I, S194L, S194M, S194F,

Subtilisin 309, Subtilisin 147, and Bacillus PB92 protease variants:

T129V, T129I, T129L, T129M, T129F
G131V, G131I, G131L, G131M, G131F
E136V, E136I, E136L, E136M, E136F,
G159V, G159I, G159L, G159M, G159F,
G164V, G164I, G164L, G164M, G164F, (BLS147)
S164V, S164I, S164L, S164M, S164F, (BLS309 AND BAPB92)
Y167A, Y167H, Y167N, Y167P, Y167C, Y167W,

N123S, T274A, N76D, V104A), in combination with any one or more of the substitutions, deletions and/or insertions mentioned above are deemed to exhibit improved properties.

Specific combinations to be mentioned are:

a) S57P+R170L
a') S57P+R170I
b) R170L+N218S
b') R170I+N218S
c) S57P+R170L+N218S
c') S57P+R170I+N218S
c") S57P+V104Y+R170L+N218S
c'") S57P+V104Y+R170I+N218S
d) R170L+N218S+M222A
d') R170I+N218S+M222S
d") R170L+N218S+M222A
d'") R170I+N218S+M222S
e) S57P+R170L+S188P+A194P
e') S57P+R170I+S188P+A194P
f) Y167L+R170L
f') Y167L+R170I
g) Y167I+R170L
g') Y167I+R170I
h) N76D+R170L+N218S
h') N76D+R170I+N218S
i) S57P+N76D+R170L+N218S
i') S57P+N76D+R170I+N218S
j) N76D+R170L+N218S+M222A
j') N76D+R170I+N218S+M222S
j") N76D+R170L+N218S+M222A
j"') N76D+R170L+N218S+M222S
k) S57P+R170I+S188P+A194P+N218S
k') S57P+R170I+S188P+A194P+N218S
l) *36D+N76D+H120D+R170L+G195E+K235L
l') *36D+N76D+H120D+R170I+G195E+K235L

bb) R170L+Y171I
bb') R170I+Y171L
bb") R170L+Y171L
bb"') R170I+Y171I
cc) Y167I+Y171L+N218S
cc') Y167I+Y171I+N218S

DETERGENT COMPOSITIONS COMPRISING THE MUTANT ENZYMES

The present invention also comprises the use of the mutant enzymes of the invention in cleaning and detergent compositions and such compositions comprising the mutant subtilisin enzymes. Such cleaning and detergent compositions can in principle have any physical form, but the subtilase variants are preferably incorporated in liquid detergent compositions or in detergent compositions in the form of bars, tablets, sticks and the like for direct application, wherein they exhibit improved enzyme stability or performance.

Among the liquid compositions of the present invention are aqueous liquid detergents having for example a homogeneous physical character, e.g. they can consist of a micellar solution of surfactants in a continuous aqueous phase, so-called isotropic liquids.

Alternatively, they can have a heterogeneous physical phase and they can be structured, for example they can consist of a dispersion of lamellar droplets in a continuous aqueous phase, for example comprising a deflocculating polymer having a hydrophilic backbone and at least one hydrophobic side chain, as described in EP-A-346 995 (Unilever) (incorporated herein by reference). These latter liquids are heterogeneous and may contain suspended solid particles such as particles of builder materials e.g. of the kinds mentioned below.

VARIOUS COMPONENTS:

1. Anionic Surfactant

The compositions of the present invention contain from about 10% to about 50%, preferably from about 15% to about 50%, more preferably from about 20% to 40%, and most preferably from 20% to about 30%, by weight of a natural or synthetic anionic surfactant. Suitable natural or synthetic anionic surfactants are *e.g.* soaps and such as disclosed in U.S. Patent 4,285,841, and in U.S. Patent 3,929,678.

Useful anionic surfactants include the water-soluble salts, particularly the alkali metal, ammonium and alkylolammonium (e.g., monoethanolammonium or triethanolammonium) salts, of organic sulfuric reaction products having in their molecular structure an alkyl group containing from about 10 to about 20 carbon atoms and a sulfonic acid or sulfuric acid ester group. (Included in the term "alkyl" is the alkyl portion of aryl groups.) Examples of this group of synthetic surfactants are the alkyl sulfates, especially those obtained by sulfating the higher alcohols (C_8-C_{16} carbon atoms) such as those produced by reducing the glycerides of tallow or coconut oil; and the alkylbenzene sulfonates in which the alkyl group contains from about 9 to about 15 carbon atoms, in straight chain or branched chain configuration, e.g., those of the type described in U. S. Patents 2,220,099 and 2,477,383. Especially valuable are linear straight chain alkylbenzene sulfonates in which the average number of carbon atoms in the alkyl group is from about 11 to 14.

Other anionic surfactants herein are the water-soluble salts of: paraffin sulfonates containing from 8 to about 24 (preferably about 12 to 18) carbon atoms; alkyl glyceryl ether sulfonates, especially those ethers of C_8-C_{18} alcohols (e.g., those

The optionally ethoxylated nonionic surfactant is of the formula $R^1(OC_2H_4)_n$ OH, wherein R^1 is a C_{10}-C_{16} alkyl group or a C_8-C_{12} alkyl phenyl group, n is from 3 to 9, and said nonionic surfactant has an HLB (Hydrophilic-Lipophilic Balance) of from 6 to 14, preferably from 10 to 13. These surfactants are more fully described in U.S. Patents 4,285,841, and 4,284,532, Particularly preferred are condensation products of C_{12}-C_{15} alcohols with from 3 to 8 moles of ethylene oxide per mole of alcohol, e.g., C_{12}-C_{13} alcohol condensed with about 6.5 moles of ethylene oxide per mole of alcohol. Other nonionic surfactants to be mentioned are APG, EGE, and glucamide surfactants.

3. Detergency Builder

Among the usual detergent ingredients which may be present in usual amounts in the detergent compositions of this invention are the following: The compositions may be built or unbuilt, and may be of the zero-P type (i.e. not containing any phosphorus containing builders). Thus, the composition may contain in the aggregate for example from 1-50%, e.g. at least about 5% and often up to about 35-40% by weight, of one or more organic and/or inorganic builders. Typical examples of builders include those already mentioned above, and more broadly include alkali metal ortho, pyro, and tripolyphosphates, alkali metal carbonates, either alone or in admixture with calcite, alkali metal citrates, alkali metal nitrilotriacetates, carboxymethyloxysuccinates, zeolites, polyacetalcarboxylates, and so on.

More specifically the compositions herein contain from 5% to 20%, preferably from 10% to 15%, by weight of a detergency builder which can be a fatty acid containing from 10 to 18 carbon atoms and/or a polycarboxylate, zeolite, polyphoshonate and/or polyphosphate a builder. Preferred are from 0 to 10% (more preferably from 3% to 10%) by weight of saturated fatty acids containing from 12 to 14 carbon atoms, along with from 0

Examples of such polycarboxylate builders are sodium and potassium ethylenediaminetetraacetate; sodium and potassium nitrilotriacetate; the water-soluble salts of phytic acid, e.g., sodium and potassium phytates, disclosed in U.S. Patent 1,739,942, the polycarboxylate materials described in U.S. Patent 3,364,103; and the water-soluble salts of polycarboxylate polymers and copolymers described in U.S. Patent 3,308,067.

Other useful detergency builders include the water-soluble salts of polymeric aliphatic polycarboxylic acids having the following structural and physical characteristics: (a) a minimum molecular weight of about 350 calculated as to the acid form; (b) an equivalent weight of 50 to 80 calculated as to acid form; (3) at least 45 mole percent of the monomeric species having at least two carboxyl radicals separated from each other by not more than two carbon atoms: (d) the site of attachment of the polymer chain of any carboxyl-containing radical being separated by not more than three carbon atoms along the polymer chain from the site of attachment of the next carboxyl-containing radical. Specific examples of such builders are the polymers and copolymers of itaconic acid, aconitic acid, maleic acid, mesaconic acid, fumaric acid, methylene malonic acid, and citraconic acid.

Other suitable polycarboxylate builders include the water-soluble salts, especially the sodium and potassium salts, of mellitic acid, citric acid, pyromellitic acid, benzene pentacarboxylic acid, oxydiacetic acid, carboxymethyloxy-succinic acid, carboxymethyloxymalonic acid, cis-cyclohexane-hexacarboxylic acid, cis-cyclopentanetetracarboxylic acid and oxydisuccinic acid.

Other polycarboxylates are the polyacetal carboxylates described in U.S. Patent 4,144,226, and U.S. Patent 4,146,495.

Expressed differently the compositions of the present invention contain from about 0.01% to about 5%, preferably from about 0.1% to about 2%, by weight of the proteolytic enzymes of the invention.

The described proteolytic enzyme is preferably included in an amount sufficient to provide an activity of from 0.05 to about 1.0, more preferably from about 0.1 to 0.75, most preferably from about 0.125 to about 0.5,mg of active enzyme per gram of composition.

The enzyme component may be added to the other components in any convenient form, such as in the form of a solution, slurry, LDP slurry, or crystals.

5. Enzyme Stabilization System

The liquid detergents according to the present invention may comprise An enzyme stabilization system, comprising calcium ion, boric acid, propylene glycol and/or short chain carboxylic acids. The enzyme stabilization system comprises from about 0.5% to about 15% by weight of the composition.

The composition preferably contains from about 0.01 to about 50, preferably from about 0.1 to about 30, more preferably from about 1 to 20 millimoles of calcium ion per liter. The level of calcium ion should be selected so that there is always some minimum level available for the enzyme, after allowing for complexation with builders etc. in the composition. Any water-soluble calcium salt can be used as the source of calcium ion, including calcium chloride, calcium formate, and calcium acetate. A small amount of calcium ion, generally from about 0.05 to 0.4 millimoles per liter, is often also present in the composition due to calcium in the enzyme slurry and formula

they contain from about 15% to about 60%, preferably from about 25% to about 45%, by weight of water.

FURTHER OPTIONAL COMPONENTS

A. Cosurfactants

Optional cosurfactants for use with the above nonionic surfactants include amides of the formula

$$R^1 - \overset{\overset{\displaystyle O}{\|}}{C} - \overset{\overset{\displaystyle R^2}{|}}{N} - R^3$$

wherein R^1 is an alkyl, hydroxyalkyl or alkenyl radical containing from 8 to 20 carbon atoms, and R^2 and R^3 are selected from the group consisting of hydrogen, methyl, ethyl, propyl, isopropyl, 2-hydroxyethyl, 2-hydroxypropyl, 3-hydroxypropyl, and said radicals additionally containing up to 5 ethylene oxide units, provided at least one of R^2 and R^3 contains a hydroxyl group.

Preferred amides are the C_8-C_{20} fatty acid alkylol amides in which each alkylol group contains from 1 to 3 carbon atoms, and additionally can contain up to 2 ethylene oxide units. Particularly preferred are the C_{12}-C_{16} fatty acid monoethanol and diethanol amides.

If used, amides are preferably present at a level such that the above ethoxylated nonionic surfactant and amide surfactant is in a weight ratio of from 4:1 to 1:4, preferably from 3:1 to 1:3.

C. Neutralization System

The present compositions can also optionally contain from about 0 to about 0.04 moles, preferably from about 0.01 to 0.035 moles, more preferably from about 0.015 to about 0.03 moles, per 100 grams of composition of an alkanolamine selected from the group consisting of monoethanolamine, diethanolamine, triethanolamine,and mixtures thereof. Low levels of the alkanolamines, particularly monoethanolamine, are preferred to enhance product stability, detergency performance, and odour. However, the amount of alkanolamine should be minimized for best chlorine bleach compatibility.

In addition, the compositions contain sodium ions, and preferably potassium ions, at a level sufficient to neutralize the anionic species and provide the desired product pH.

D. Suds Suppressor

Another optional component for use in the liquid detergents herein is from 0 to about 1.5%, preferably from about 0.5% to about 1.0%, by weight of silicone based suds suppressor agent. Silicones are widely known and taught for use as highly effective suds controlling agents. For example, U.S. Patent 3,455,839 relates to compositions and processes for defoaming aqueous solutions by incorporating therein small amounts of polydimethylsiloxane fluids.

Useful suds controlling silicones are mixtures of silicone and silanated silica as described, for instance, in German Patent Application DOS 2,124,526.

Silicone defoamers and suds controlling agents have been successfully incorporated into granular detergent compositions

(e.g. as in EP Patent Publication No. 258,068 (Novo Nordisk A/S)

The added amount of lipase can be chosen within wide limits, for example 50 to 30,000 LU/g per gram of the surfactant system or of the detergent composition, e.g. often at least 100 LU/g, very usefully at least 500 LU/g, sometimes preferably above 1000, above 2000 LU/g or above 4000 LU/g or more, thus very often within the range of 50-4000 LU/g, and possibly within the range of 200-1000 LU/g. In this specification, lipase units are defined as they are in EP Patent Publication No. 258,068.

The lipolytic enzyme can be chosen among a wide range of lipases. In particular, the lipases described in for example the following patent specifications: EP Patent Publications Nos. 214,761 (Novo Nordisk A/S), 258,068, and especially lipases showing immunological cross reactivity with antisera raised against lipase from *Thermomyces lanuginosus* ATCC 22070, EP Patent Publications Nos. 205,208 and 206,390, and especially lipases showing immunological cross-reactivity with antisera raised against lipase from *Chromobacter viscosum* var lipolyticum NRRL B-3673, or against lipase from *Alcaligenes* PL-679, ATCC 31371 and FERM-P 3783, also the lipases described in specifications WO 87/00859 (Gist-Brocades) and EP Patent Publication No. 204,284 (Sapporo Breweries). Suitable, in particular, are for example the following commercially available lipase preparations: Lipolase® Novo Nordisk A/S, Amano lipases CE, P, B, AP, M-AP, AML, and CES, and Meito lipases MY-30, OF, and PL, also Esterase® MM (Novo Nordisk A/S), Lipozym, SP225, SP285, (all Novo Nordisk A/S) Saiken lipase, Enzeco lipase, Toyo Jozo lipase and Diosynth lipase (Trade Marks), Lumafast® (Genencor Inc.), Lipomax® (Gist-Brocades N.V.), and lipases as described in WO 94/03578 (Unilever).

Specific examples are 2-dodecenyl succinate (preferred) and 2-tetradecenyl succinate.

The compositions herein optionally contain from about 0.1% to about 1%, preferably from about 0.2% to about 0.6%, by weight of water-soluble salts of ethylenediamine tetramethylenephosphonic acid, diethylenetriamine pentamethylenephosphonic acid, ethylenediamine tetraacetic acid (preferred), or diethylenetriamine pentaacetic acid (most preferred) to enhance cleaning performance when pretreating fabrics.

Furthermore, the detergent compositions may contain from 1-35% of a bleaching agent or a bleach precursor or a system comprising bleaching agent and/or precursor with activator therefor.

Further optional ingredients are lather boosters, anti-corrosion agents, soil-suspending agents, sequestering agents, anti-soil redeposition agents, and so on.

The compositions herein preferably contain up to about 10% of ethanol.

G. Other Properties

The instant composition usually has a pH, in a 10% by weight solution in water at 20°C, of from about 7.0 to 9.0, preferably from about 8.0 to about 8.5.

The instant compositions can also have a Critical Micelle Concentration (CMC) of less than or equal to 200 parts per million (ppm), and an air/water Interfacial Tension above the CMC of less than or equal to 32, preferably less than or equal to about 30, dynes per centimetre at 35°C in distilled water. These measurements are described in "Measurement of Interfacial Tension and Surface Tension - General Review for Practical Man"

c) An enzymatic liquid detergent composition formulated to give a wash liquor pH of 9 or less when used at a rate corresponding to 0.4-0.8 g/l surfactant.

d) An enzymatic liquid detergent composition formulated to give a wash liquor pH of 8.5 or more when used at a rate corresponding to 0.4-0.8 g/l surfactant.

e) An enzymatic liquid detergent composition formulated to give a wash liquor ionic strength of 0.03 or less, e.g. 0.02 or less, when used at a rate corresponding to 0.4-0.8 g/l surfactant.

f) An enzymatic liquid detergent composition formulated to give a wash liquor ionic strength of 0.01 or more, e.g. 0.02 or more, when used at a rate corresponding to 0.4-0.8 g/l surfactant.

It was found that the subtilase variants of the present invention can also be usefully incorporated in detergent composition in the form of bars, tablets, sticks and the like for direct application to fabrics, hard surfaces or any other surface. In particular, they can be incorporated into soap or soap/synthetic compositions in bar form, wherein they exhibit a remarkable enzyme stability. Detergent composition in the form of bars, tablets, sticks and the like for direct application, are for example described in South African Patent 93/7274, incorporated herein by reference.

Accordingly, the preferred bars in accordance with this invention comprise, in addition to the subtilase variant:

i) 25 to 80%, most preferably 25 to 70%, by weight of detergent active which is soap or a mixture of soap and synthetic detergent active, reckoned as anhydrous;

Yet another method for identifying subtilase-producing clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming protease-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for subtilase, such as skim milk. Those bacteria containing subtilase-bearing plasmid will produce colonies surrounded by a halo of clear agar, due to digestion of the skim milk by excreted subtilase.

GENERATION OF RANDOM MUTATIONS IN THE SUBTILASE GENE

Once the subtilase gene has been cloned into a suitable vector, such as a plasmid, several methods can be used to introduce random mutations into the gene.

One method would be to incorporate the cloned subtilase gene, as part of a retrievable vector, into a mutator strain of *Eschericia coli*.

Another method would involve generating a single stranded form of the subtilase gene, and then annealing the fragment of DNA containing the subtilase gene with another DNA fragment such that a portion of the subtilase gene remained single stranded. This discrete, single stranded region could then be exposed to any of a number of mutagenizing agents, including, but not limited to, sodium bisulfite, hydroxylamine, nitrous acid, formic acid, or hydralazine. A specific example of this method for generating random mutations is described by Shortle and Nathans (1978, *Proc. Natl. Acad. Sci. U.S.A.*,75 2170-2174). According to the shortle and Nathans method, the plasmid bearing the subtilase gene would be nicked by a restriction enzyme that cleaves within the gene. This nick would be widened into a gap using the exonuclease action of DNA polymerase I. The resulting single-stranded gap could then be mutagenized using any one of the above mentioned mutagenizing agents.

D strain of E. coli which increases the range of mutations due to its error prone DNA polymerase.

The mutagens nitrous acid and formic acid may also be used to produce mutant libraries. Because these chemicals are not as specific for single-stranded DNA as sodium bisulfite, the mutagenesis reactions are performed according to the following procedure. The coding portion of the subtilisin gene is cloned in M13 phage by standard methods and single stranded phage DNA prepared. The single-stranded DNA is then reacted with 1 M nitrous acid pH. 4.3 for 15-60 minutes at 23°C or 2.4 M formic acid for 1-5 minutes at 23°C. These ranges of reaction times produce a mutation frequency of from 1 in 1000 to 5 in 1000. After mutagenesis, a universal primer is annealed to the M13 DNA and duplex DNA is synthesized using the mutagenized single-stranded DNA as a template so that the coding portion of the subtilisin gene becomes fully double-stranded. At this point the coding region can be cut out of the M13 vector with restriction enzymes and ligated into an un-mutagenized expression vector so that mutations occur only in the restriction fragment. (Myers *et al.*, *Science* **229** 242-257 (1985)).

GENERATION OF SITE DIRECTED MUTATIONS IN THE SUBTILASE GENE

Once the subtilase gene has been cloned, and desirable sites for mutation identified and the residue to substitute for the original ones have been decided, these mutations can be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a preferred method, a single stranded gap of DNA, bridging the subtilase gene, is created in a vector bearing the subtilase gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then

purposes. An expression vector includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes. To permit the secretion of the expressed protein, nucleotides encoding a "signal sequence" may be inserted prior to the coding sequence of the gene. For expression under the direction of control sequences, a target gene to be treated according to the invention is operably linked to the control sequences in the proper reading frame. Promoter sequences that can be incorporated into plasmid vectors, and which can support the transcription of the mutant subtilase gene, include but are not limited to the prokaryotic ß-lactamase promoter (Villa-Kamaroff, *et al.* (1978) *Proc. Natl. Acad. Sci. U.S.A.* 75 3727-3731) and the tac promoter (DeBoer, *et al.* (1983) *Proc. Natl. Acad. Sci. U.S.A.* 80 21-25). Further references can also be found in "*Useful proteins from recombinant bacteria*" in *Scientific American* (1980) 242 74-94.

According to one embodiment *B. subtilis* is transformed by an expression vector carrying the mutated DNA. If expression is to take place in a secreting microorganism such as *B. subtilis* a signal sequence may follow the translation initiation signal and precede the DNA sequence of interest. The signal sequence acts to transport the expression product to the cell wall where it is cleaved from the product upon secretion. The term "control sequences" as defined above is intended to include a signal sequence, when it is present.

Other host systems known to the skilled person are also contemplated for the expression and production of the protease variants of the invention. Such host systems comprise fungi, including filamentous fungi, plant, avian and mammalian cells, as well as others.

Rothgeb, T.M., Goodlander, B.D., Garrison, P.H., and Smith, L.A., (1988).

EXAMPLES

For the generation of enzyme variants according to the invention the same materials and methods as described in *i.a.* WO 89/06279 (Novo Nordisk A/S), EP 130,756 (Genentech), EP 479,870 (Novo Nordisk A/S), EP 214,435 (Henkel), WO 87/04461 (Amgen), WO 87/05050 (Genex), EP application no. 87303761 (Genentech), EP 260,105 (Genencor), WO 88/06624 (Gist-Brocades NV), WO 88/07578 (Genentech), WO 88/08028 (Genex), WO 88/08033 (Amgen), WO 88/08164 (Genex), Thomas *et al.* (1985) *Nature*, **318** 375-376; Thomas *et al.* (1987) *J. Mol. Biol.*, 193, 803-813; Russel and Fersht (1987) *Nature* **328** 496-500. Other methods well established in the art may also be used.

EXAMPLE 1

Construction and Expression of Enzyme Variants:

A vector suited to a synthetic gene coding for subtilase 309 and its mutants was constructed. It is essentially a pUC19 plasmid [Yanish-Perron and Messing (1985) *Gene;* **33** 103-119], in which the multiple cloning site has been replaced by a linker containing the restriction sites used to separate five sub--fragments constituting the gene. The new linker was inserted into EcoRI - HindIII cut pUC19 thereby destroying these sites. The details of this construction are described in WO 92/19729 on pages 25-26 and in figure 1 (sheets 1/7-7/7) thereof, the content of which is hereby included by reference.

Each subfragment was made from 6 to 12 oligonucleotides. The oligonucleotides were synthesised on an automatic DNA

92/19729), heated to 100°C, cooled to 0°C, and transformed into *E. coli*. After retransformation, the recombinants can be screened by colony hybridisation using 32-P-labelled NOR 789. The recombinants that turned out to be positive during the screening had the KpnI site introduced right in front of NcoI by changing two bases without changing the amino acid sequence. pSX172 is described in EP Patent Publication No. 405 901. The KpnI site so created is inserted into pSX120 on a 400-bp PvuI-NheI fragment, giving rise to pSX212. pSX120 is also described in EP Patent Publication No. 405 901.

The synthetic gene is inserted between KpnI and BamHI on pSX212, giving rise to pSX222.

Examples of mutations and corresponding sequences of oligonucleotides are as follows:

<u>R170L</u> (fragment D1)

```
5`- AATTCAGGTGCAGGCTCAATCAGCTATCCGGCGCTCTAT - 3`
        ||||||||||||||||||||||||||||||*||||
5`-     GTCCACGTCCGAGTTAGTCGATAGGCCGCGAGATACGCTTG -3`
```

<u>R170I</u> (fragment D1)

```
5`- AATTCAGGTGCAGGCTCAATCAGCTATCCGGCGATCTAT - 3`
        |||||||||||||||||||||||||||||**||||
5`-     GTCCACGTCCGAGTTAGTCGATAGGCCGCTAGATACGCTTG -3`
```

<u>S57P</u> (fragment B1)

```
5' - AGCTTTGTACCAGGGGAACCGCCGACTCAAGATGGG - 3'
         |||||||||||||||||*||||||||||||||
3' - AACATGGTCCCCTTGGCGGCTGAGTTCTACCCTTACCC - 5'
```

These oligonucleotides were combined with the rest of the oligonucleotides from the synthetic gene that was not changed.

The protease was eluted using a linear gradient of 0-0.1 M sodium chloride in 2 litres of the same buffer (0-0.2 M sodium chloride in case of Subtilisin 147).

In a final purification step protease containing fractions from the CM Sepharose column were combined and concentrated in an Amicon ultrafiltration cell equipped with a GR81PP membrane (from the Danish Sugar Factories Inc.).

By using the techniques of Example 1 for the construction and the above isolation procedure the following subtilisin 309 variants were produced and isolated:

A: G159I
B: S164I
C: Y167I
D: R170I
E: R170L
F: R170M
G: R170F
H: G195F
I: S57P+R170L
J: R170L+N218S
K: S57P+R170L+N218S
L: R170L+N218S+M222A
M: S57P+R170L+S188P+A194P
N: Y167I+R170L
O: S57P+R170L+Q206E
P: R170L+Q206E
Q: Y167I+R170L+Q206E
R: Y167I+R170L+A194P
S: Y167I+R170L+N218S
T: Y167I+R170L+A194P+N218S
U: Y167I+Y171I
V: R170G

Ingredient	%
NaLAS	8.0
Neodol 25-9	8.0
AES 25-3S	14.0
NaCitrate.$2H_2O$	5.0
Propylene Glycol	5.0
Sorbitol	4.5
F-dye Tinopal UNPA-GX	0.15
Lytron 614 Opacifier	0.03
Kathon Preservative	0.0003
Acid Blue 80	0.00117
Acid Violet 48	0.0033
SAVINASE® 16L	0.25
LIPOLASE® 100L	0.70
Fragrance	0.15
Water	ad 100.0

The pH is adjusted to 7.1.

Table III.

Residual enzyme activity (in percentage of original activity) after storage at 37°C for Example D1 comprising the BLS309 variant S57P+R170L+N218S.

Example D2:

A non-aqueous detergent liquid according to an embodiment of the invention is formulated using 38.5% C13-C15 linear primary alcohol alkoxylated with 4.9 mol/mol ethylene oxide and 2.7 mol/mol propylene oxide, 5% triacetin, 30% sodium triphosphate, 4% soda ash, 15.5% sodium perborate monohydrate containing a minor proportion of oxoborate, 4% TAED, 0.25% EDTA of which 0.1% as phosphonic acid, Aerosil 0.6%, SCMC 1%, and 0.6% protease. The pH is adjusted to a value between 9 and 10, e.g. about 9.8.

Example D3:

Structured liquid detergents can for example contain, in addition to a protease as described herein, 2-15% nonionic surfactant, 5-40% total surfactant, comprising nonionic and optionally anionic surfactant, 5-35% phosphate-containing or non-phosphate containing builder, 0.2-0.8% polymeric thickener, e.g. cross-linked acrylic polymer with m.w. over 10^6, at least 10% sodium silicate, e.g. as neutral waterglass, alkali (e.g. potassium-containing alkali) to adjust to desired pH, preferably in the range 9-10 or upwards, e.g. above pH 11, with a ratio sodium cation: silicate anion (as free silica) (by weight) less than 0.7:1, and viscosity of 0.3-30 Pas (at 20°C and 20^{s}-^{1}).

Suitable examples contain about 5% nonionic surfactant C13-15 alcohol alkoxylated with about 5 EO groups per mole and with about 2.7 PO groups per mole, 15-23% neutral waterglass with 3.5 weight ratio between silica and sodium oxide, 13-19% KOH, 8-23% STPP, 0-11% sodium carbonate, 0.5% Carbopol 941 (TM).

Protease may be incorporated at for example 0.5%.

Table VI

Storage (days)	Enzyme	WT	R170M	S57P+R170L +Q206E	Y167I+R170L+ N218S
0		100	100	100	100
0.1		90.2	78	97	94
1		58	53	95	68
2		40	34	87	55
5		16	27	75	29
6		12	22	73	24
8		8	19	77	17
14		2	11	52	4

From Table VI it can be seen that the variants tested exhibit improved stability in comparison to the wild type enzyme in this type of detergent

Example D6:

Soap bars were produced containing 49.7 wt. 80/20 tallow /coconut soap, 49.0% water, 20% sodium citrate, 1.0% citric acid and 0.031% protease. After preparation of the soap bars they were stored at ambient temperature and after specific time intervals samples were taken and measured for protease activity. The stability data are given below:

Table VIII

Storage (days)	Enzyme	WT	R170L	R170L+N218S+S57P	R170L+Y167I
0		100	100	100	100
1		50	100	97	94
2		25	91	100	83
3		-	100	94	80
6		-	98	89	90
10		0	100	94	71
17		-	93	80	73
27		-	95	86	70

From the above Table it is evident that the subtilase variants R170L, R170L+N218S+S57P and R170L+Y167I exhibit a remarkably improved stability in this type of detergent.

Example D7:

Soap bars were produced containing 63.88% 80/20 tallow/coconut soap, 1% coconut fatty acid, 25.1% water, 10% sodium citrate and 0.021% protease. The laundry soap bars were stored at 37°C

Experimental conditions

Table X: Experimental conditions for evaluation of Subtilisin 309 variants.

Detergent	Protease model detergent '95
Detergent dose	3 g/l
pH	9.5
Wash time	15 min.
Temperature	15°C
Water hardness	9°dH ~ 1.61 mM Ca^{2+}/Mg^{2+}
Enzymes	Subtilisin 309 variants as listed below
Enzyme conc.	0; 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0, 3.0 mg/l
Test system	150 ml beakers with a stirring rod.
Cloth/volume	5 cloths (Ø 2.5 cm) / 50 ml Detergent solution.
Cloth	Cotton soiled with grass juice

Subsequent to washing the cloths were flushed in tap water and air-dried.

The above model detergent is a simple detergent formulation. The most characteristic features are that STP is used as builder and the content of anionic tenside (LAS) is quite high. Further the pH is adjusted to 9.5, which is low for a powder detergent.

Table XII: Variants and improvement factors for Subtilisin 309.

Designation	Variant	IF
S003*	R170Y	2.8
S004*	R170Y + G195E	2.6
S012*	R170Y + G195E + K251E	1.6
G	R170F	3.3
E	R170L	3.8
F	R170M	2.4
D	R170I	4.1
I	S57P + R170L	3.9
J	R170L + N218S	1.6
K	S57P + R170L + N218S	2.3
N	Y167I + R170L	6.2
P	R170L + Q206E	2.6
V	R170G	2.0
W	R170C	3.4
O	S57P + R170L + Q206E	2.9
Q	Y167I + R170L + Q206E	2.4
R	Y167I + R170L + A194P	5.1
X	Y171I	1.2
Y	Y167I + R170L + N218S	4.0
T	Y167I + R170I + A194P + N218S	3.6

* Described in WO 91/00345

As it can be seen from Table XII all the Subtilisin 309 variants of the invention exhibits an improvement in wash performance.

PATENT CLAIMS

1. A subtilase variant having improved storage stability and/or improved performance in detergents, wherein one or more amino acid residues situated in, or in the vicinity of a hydrophobic domain of the parent subtilase have been substituted for an amino acid residue more hydrophobic than the original residue, said hydrophobic domain comprising the residues corresponding to residues P129, P131, I165, Y167, Y171 of BLS309 (in BASBPN numbering), and said residues in the vicinity thereof comprises residues corresponding to the residues E136, G159, S164, R170, A194, and G195 of BLS309 (in BASBPN numbering), with the exception of the R170M, R170I and R170V variants of BABP92 and the R170Y variant of BLS309.

2. The variant of claim 1, wherein said variant exhibits improved stability in liquid detergents.

3. The variant of claim 1, wherein said variant exhibits improved stability in detergents in shaped solid form.

4. The variant of claim 1, wherein said variant exhibits improved wash performance.

5. The variant of any of the claims 1 to 4, wherein the original amino acid residue has been substituted for any other residue which increase the hydrophobicity of the original residue, where the substituted residue preferably is a residue selected from the group comprising Val (V), Ile (I), Leu (L), Met (M), Phe (F), Pro (P), and Trp(W), especially Val, Ile or Leu.

6. The variant of any of the claims 1 to 5, wherein the parent subtilase is chosen from the sub-group I-S1.

Y170V, Y170I, Y170L, Y170M, Y170F,
Y171A, Y171H, Y171N, Y171P, Y171C, Y171W,
Y171Q, Y171S, Y171T, Y171G
Y171V, Y171I, Y171L, Y171M, Y171F
S194V, S194I, S194L, S194M, S194F,
E136V, E136I, E136L, E136M, E136F,
G159V, G159I, G159L, G159M, G159F,
G164V, G164I, G164L, G164M, G164F,
S164V, S164I, S164L, S164M, S164F,
Y167A, Y167H, Y167N, Y167P, Y167C, Y167W,
Y167Q, Y167S, Y167T, Y167G
Y167V, Y167I, Y167L, Y167M, Y167F
R170A, R170H, R170N, R170P, R170C, R170W
R170Q, R170S, R170T, R170Y, R170G
R170V, R170I, R170L, R170M, R170F,
A194V, A194I, A194L, A194M, A194F,
P194V, P194I, P194L, P194M, P194F,
E195V, E195I, E195L, E195M, E195F,
G195V, G195I, G195L, G195M, G195F,

14. The variant of any of claims 1 to 13, wherein said variant is combined with further substitutions, deletions and/or insertions in any one or more of the positions: 27, 36, 57, 76, 97, 101, 104, 120, 123, 206, 218, 222, 224, 235 and 274.

15. The variant of claim 14, wherein said subtilase belongs to the I-S2 sub-group and said further change is chosen from the group comprising K27R, *36D, S57P, N76D, G97N, S101G, V104A, V104N, V104Y, H120D, N123S, A194P, Q206E, N218S, M222S, M222A, T224S, K235L, and T274A.

16. The variant of claim 15 comprising any one or two of the substitutions X167V, X167M, X167F, X167L, X167I, X170V, X170M, X170F, X170L, and/or X170I in combination with any one

j) N76D+R170L+N218S+M222A

j') N76D+R170I+N218S+M222S

j") N76D+R170L+N218S+M222A

j"') N76D+R170L+N218S+M222S

k) S57P+R170I+S188P+A194P+N218S

k') S57P+R170I+S188P+A194P+N218S

l) *36D+N76D+H120D+R170L+G195E+K235L

l') *36D+N76D+H120D+R170I+G195E+K235L

l") *36D+N76D+H120D+Y167I+R170L+G195E+K235L

l"') *36D+N76D+H120D+Y167I+R170I+G195E+K235L

m) N76D+H120D+R170L+G195E+K235L

m') N76D+H120D+R170I+G195E+K235L

m") N76D+H120D+Y167I+R170L+G195E+K235L

m"') N76D+H120D+Y167I+R170I+G195E+K235L

n) *36D+G97N+V104Y+H120D+R170L+A194P+G195E+K235L

n') *36D+G97N+V104Y+H120D+R170I+A194P+G195E+K235L

o) S57P+R170L+Q206E

o') S57P+R170I+Q206E

p) R170L+Q206E

p') R170I+Q206E

q) Y167I+R170L+Q206E

q') Y167I+R170I+Q206E

r) Y167F+R170L

r') Y167F+R170I

t) Y167I+R170L+A194P

t') Y167I+R170I+A194P

t") Y167L+R170L+A194P

t"') Y167L+R170I+A194P

u) Y167I+R170L+N218S

u') Y167I+R170I+N218S

u") Y167L+R170L+N218S

u"') Y167L+R170I+N218S

v) Y167I+R170L+A194P+N218S

v') Y167I+R170I+A194P+N218S

v") Y167L+R170L+A194P+N218S

22. A vector comprising a DNA sequence of claim 21.

23. A microbial host transformed with a vector of claim 22.

24. The microbial host of claim 23, which is a bacterium, preferably a Bacillus.

25. The microbial host of claim 23, which is a fungus or yeast, preferably a filamentous fungus, especially an Aspergillus.

26. A method for producing a variant of any of claims 1 to 18, wherein a host of any of claims 23 to 25 is cultured under conditions conducive to the expression and secretion of said variant, and the variant is recovered.

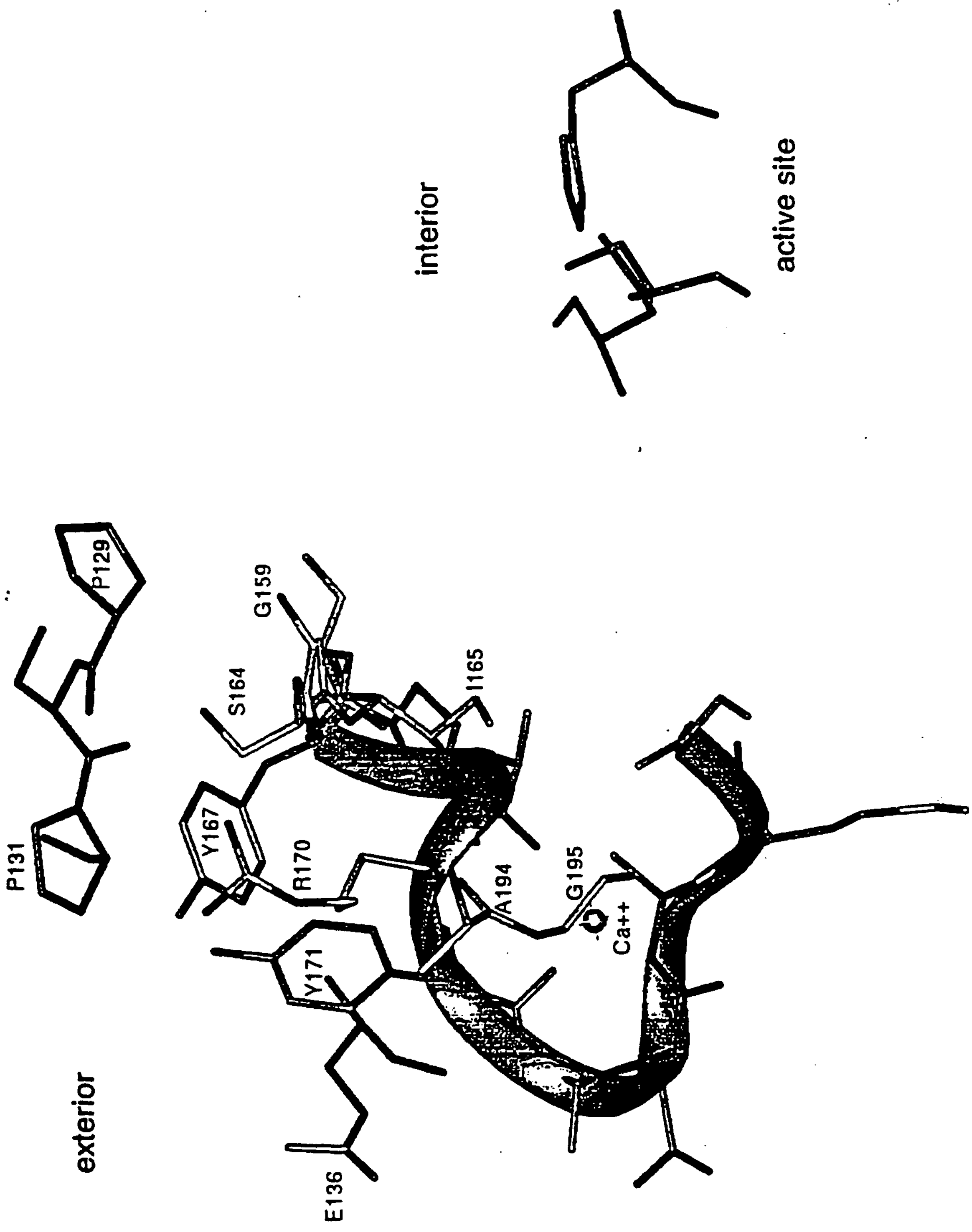
interior
active site
P129
G159
S164
I165
P131
Y167
R170
A194
G195
Ca++
Y171
exterior
E136

FIG. 2

INTERNATIONAL SEARCH REPORT

Information on patent family members

01/07/96

International application No.
PCT/DK 96/00207

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